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Point of VIEW

Metal Sensing by RNA in Bacteria: Exception or Rule?

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ABSTRACT Until 2006, it was believed that bacteria control their intracellular metal ion concentrations exclusively through metal-sensing proteins. However, the detection of the first riboswitch that uses a small ion, Mg^{2+}, as ligand to regulate gene expression in *Salmonella* challenged this assumption. Now, the discovery and in-depth characterization of a second metal-ion-sensing riboswitch that seems to be ubiquitously present in Gram-positive bacteria suggest that metal sensing by RNAs may represent a widespread mechanism.

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THE RIBOSWITCH CONCEPT

The term riboswitch was coined in 2002, when two laboratories independently discovered that binding of small metabolites to messenger RNA (mRNA) leader regions can mediate bacterial gene regulation (*1, 2*). All riboswitches comprise a receptor (aptamer) region characterized by a consensus sequence/structure, which specifically binds the substrate molecule, and an output (expression platform) region. Binding of the ligand triggers a conformational switch that alters gene expression by one of three possible mechanisms: premature transcription termination (transcription attenuation), inhibition of translation initiation, or mRNA cleavage (reviewed in ref 3).

The thiamine riboswitch (*1*) is a so-called translational riboswitch, commonly found in Gram-negative bacteria (see Figure 1, panel a). Ligand (thiamine pyrophosphate, TPP) binding inhibits translation through sequestration of the Shine–Dalgarno (SD) sequence. By contrast, the riboflavin (RFN) or flavin mononucleotide (FMN) riboswitch (*2*) works by transcription attenuation, a mechanism mostly found in Gram-positive bacteria. Here, alternative RNA folding is induced by binding of a ligand (FMN) to the leader region of nascent riboflavin mRNA (see Figure 1, panel b). Over the past five years, a remarkable variety of riboswitches has been discovered that use ~12 different small ligands (*4*). Riboswitches that bind FMN, TPP, *S*-adenosyl-methionine (SAM), vitamin B_{12}, lysine, or guanine have been found to regulate the expression of a multitude of genes encoding metabolic enzymes. Whereas in all these riboswitches metabolite binding decreases gene expression, both the adenine and the glycine riboswitches work as activators of gene expression by inducing a transcriptional readthrough conformation. The peculiarity of the glycine riboswitch lies in its use of twin aptamer domains. In the case of sulfur and methionine metabolism, three different riboswitch classes have been found that contain an S-box, an S_{MK} box, or a SAM-II box (reviewed in ref 4).

Even though numerous cases of metabolite-sensing riboswitches had been validated and had indicated the versatility of such mechanisms, a surprising finding was that even tiny metal ions, such as Mg^{2+}, can directly promote control by acting as ligands (*5, 6*). Hitherto, crystal structures of the metabolite binding domains of three riboswitches responding to purines, TPP, and SAM have been solved (reviewed in ref *7*). These structures provide the foundation for a detailed mechanistic understanding of riboswitch-mediated modulation of gene expression.

A New Metal-Sensing Riboswitch: Discovery, Characterization, and Mechanism of Action. Recently, the Winkler group reported the structure and mechanism of a new metal-sensing RNA found upstream of the *mgtE* gene of *Bacillus subtilis*, designated M-box (*8*). This study is exciting for two reasons: first, it not only describes a new riboswitch using a metal-ion

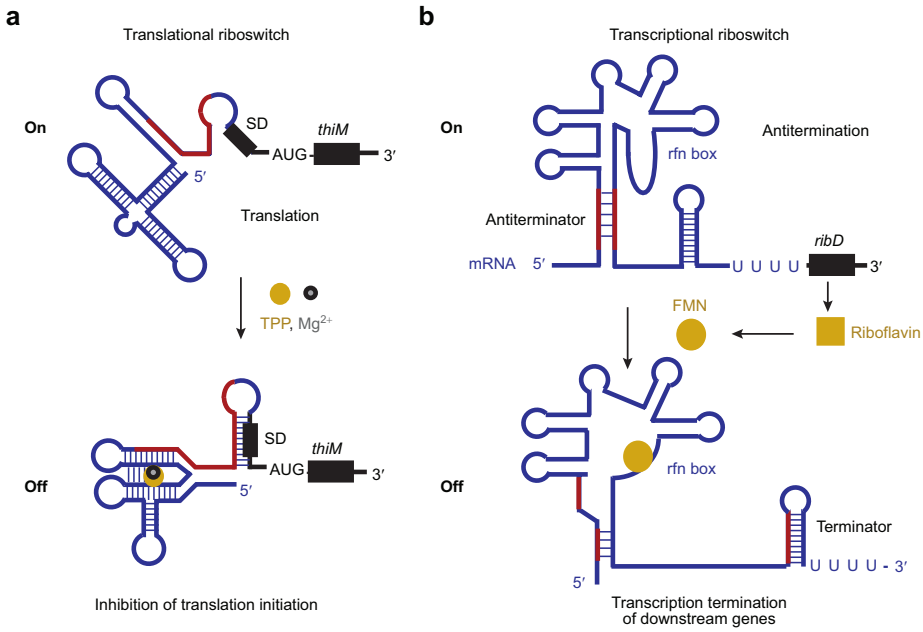

Figure 1. A schematic representation of the two principal types of riboswitches. Blue denotes aptamer, red sequences involved in alternative base-pairing, black expression platform, and yellow circles ligands. On and Off indicate conditions where the expression of downstream genes is increased or reduced, respectively. a) Translational riboswitch (TPP riboswitch of *E. coli*). In the absence of the metabolite TPP, the 5′ UTR of *thiM* mRNA folds into a structure that allows binding of the ribosomes to the SD sequence and, hence, translation of *thiM*. Binding of TPP and Mg^{2+} to the *thi* box induces an alternative conformation that sequesters the SD sequence, preventing ribosome binding and, consequently, translation of *thiM*. b) Transcriptional riboswitch (riboflavin riboswitch of *B. subtilis*). In the absence of the metabolite FMN, the leader region of the *ribD*-mRNA folds into a structure facilitating formation of a transcriptional antiterminator. Binding of FMN to the RFN box induces an alternative fold, which disrupts the antiterminator and facilitates formation of the terminator leading to premature transcription termination and shut-off of riboflavin biosynthesis.

ligand, but it also combines its detailed molecular and biochemical analyses with its structural characterization, the latter of which offers an insight into the mechanism of riboswitch action. Second, the M-box aptamer motif seems to be quite common among Gram-positive bacteria, an indication that metal sensing by RNAs is widespread rather than exceptional.

By computational approaches, several RNA elements that share properties with well-characterized riboswitches but whose ligands were unknown have been detected upstream of known and unknown genes. Among those is the region upstream of the *mgtE* gene in *B. subtilis*, previously termed the *ykoK* element but now renamed M-box. This motif was found upstream of Mg^{2+} transport genes in Gram-positive bacteria. A series of methods was used to characterize this RNA element in detail: *mgtE–lacZ* reporter gene fusions were shown to be repressed specifically by increasing the intracellular concentration of Mg^{2+}. Computer-predicted transcription terminator and antiterminator, features characteristic for a transcriptional riboswitch (see Figure 1, panel b), were experimentally confirmed. Transcription attenuation was studied both *in vivo* and *in vitro* in the presence and absence of Mg^{2+} and other ions. Analytical ultracentrifugation demonstrated that Mg^{2+} induces a compacted conformation of the M-box element. Hydroxyl radical footprinting revealed Mg^{2+}-dependent protections and confirmed the formation of a closely packed internal core. The authors concluded that the aptamer domain is dominated by a secondary structure in the presence of low Mg^{2+} but is substantially rearranged through tertiary interactions upon Mg^{2+} association (*8*).

The crystal structure of the M-box was solved in its ligand-bound state, that is, at 10 mM Mg^{2+}, at 2.6 Å (*8*). Under these conditions, the RNA adopts a 3D structure composed of three closely packed nearly parallel helices, P4, P5, and P2, which form an internal core (Figure 2, panel b). The stretches with decreased internucleotide flexibility in response to Mg^{2+} are located in the region where the three parallel helices build up a network of long-range contacts. The most exciting aspect is the presence of six Mg^{2+} ions predominantly in this domain of tertiary contacts. In addition, four K^{+} ions were also modeled in the structure, although they are dispensable for the formation of the compact conformation. Of the six Mg^{2+}, Mg1 is the key organizer of the riboswitch tertiary structure: it exhibits four inner-sphere RNA contacts to L5 nonbridging phosphate oxygens and multiple outer-shell RNA contacts to L5 positions through two water molecules. Mg2 coordinates two nonbridging phosphate oxygens in L5 and P2, bringing the parallel P5 and P2 helices together. Mg3 stabilizes the local structure, allowing for a long-range base interaction between positions P2 and L5. In contrast to these three key metal ions, Mg4–6 play only a minor role. Four evolutionarily conserved A-minor motifs, characterized by an adenosine that fits into the minor groove of

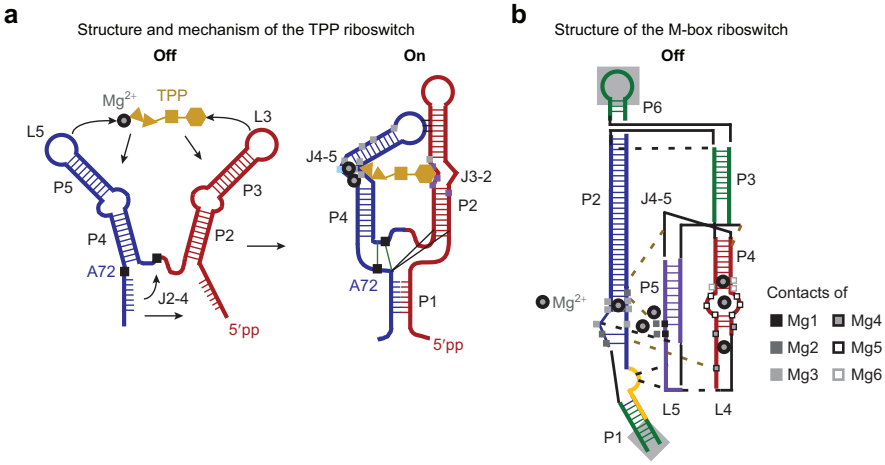

Figure 2. Schematic representation of two riboswitch structures. On and Off indicate conditions where the expression of downstream genes is increased or reduced, respectively. a) TPP riboswitch. The TPP binding pocket is formed by two parallel helices, a pyrophosphate binding helix (blue, comprising P4, J4-5, P5, and L5) and a pyrimidine binding helix (red, comprising P2, J3-2, P3, and L3). P1 is only formed in the presence of TPP. TPP is shown in yellow, Mg^{2+} is shown in gray, nonstandard base pairs are shown in green, and hydrogen bonds are shown by black lines. Light blue (G78) and magenta boxes (G40, G42, A43, and G19) symbolize bases contacted by the pyrophosphate and the pyrimidine moiety of TPP, respectively. Gray boxes indicate the binding sites for the four Mg^{2+} ions. Only the two Mg^{2+} that are involved in the pyrophosphate contact with J4-5 are shown as gray or black circles. The other Mg^{2+} ions are required for folding. TPP binding stabilizes the three-way junction with A72, thus helping the sensor domain to sequester the segment that would otherwise participate in the alternative structure of the expression platform. b) M-box riboswitch. The three parallel helices, P2, P5, and P4, are shown in blue, magenta, and red, respectively. The other helices are drawn in green. Yellow denotes the antiterminator. Gray-shaded regions indicate sequences added for crystallization. Black dotted lines indicate long-range interactions. Brown dotted lines indicate A-minor motifs. Gray and black circles symbolize the six Mg^{2+} ions coordinated to RNA. The contacts exerted by each individual Mg^{2+} are shown by small rectangles as described in the inset. For simplicity, inner- and outer-sphere contacts are not differentiated. Based on Figure 5, panel a, and Figure 6, panel a, from ref 8.

a Watson–Crick base pair, play a significant role in reducing the energetic cost of bringing the three parallel helices into proximity. These structural data explain how intracellular Mg^{2+} affects the formation of the mutually exclusive terminator or antiterminator helices: at high Mg^{2+}, helix P1 is preferentially stabilized, because several antiterminator positions are sequestered by Mg^{2+} into tertiary structure by base triples and base stacking within the L4–L5–P2 structure. This leads to transcription termination. At low Mg^{2+}, the alternative antiterminator helix can form. Thus, the main principle of the M-box riboswitch mechanism is metal-induced occlusion of antiterminator nucleotides. Consequently, it is not surprising that the most conserved positions in M-box RNAs are those that interact with metals and assist in coordinating the L4–L5–P2-tripartite structure (*8*).

Structures and Ligand Binding by Other Riboswitches. Two crystal structures of purine riboswitches have been solved (*9, 10*). Similar to the hammerhead ribozyme, the purine riboswitch folds into three helices, P1, P2, and P3, arranged as an inverted "h". The purine ligand is buried in a solvent-inaccessible pocket at the three-way junction of P1–P3 and is primarily recognized by pyrimidine residue 74 of the riboswitch by a Watson–Crick interaction. This enables the same RNA scaffold to discriminate between adenine and guanine by using U74 or C74, respectively (summarized in ref *7*).

The TPP riboswitch of *Escherichia coli* (*11, 12*) adopts a compact inverted-h architecture with two parallel sets of coaxially stacked helices (P1–P2–P3 and P4–P5) joined by a three-way junction (Figure 2, panel a). Unlike purine, TPP is not buried at the three-way junction; it instead bridges the two parallel helical stacks that separately recognize the aminopyrimidine and the pyrophosphate moieties of TPP (see Figure 2, panel a). The pyrimidine sensor helix is largely preformed, whereas the pyrophosphate sensor helix becomes organized concomitant with ligand binding.

The SAMI-riboswitch structure (*13*) is distinctly different: it contains two stacks (P1–P4 and P2a–P3) that cross at an angle of ~70°. A pseudoknot appears to stabilize this fold. Like TPP, SAM bridges two helical stacks. P1 recognizes the ribose–sulfur backbone of SAM, P3 binds the adenine ring and amino acid *via* hydrogen bonds and stacking interactions, whereas the sulfur atom makes an electrostatic interaction.

However, despite the three available crystal structures of metabolite binding domains, the elucidation of the molecular mechanisms by which the different riboswitches work is still in its infancy. For transcriptional riboswitches, cotranscriptional mRNA folding is essential for their mechanism of action and must be considered alongside the thermodynamics and kinetics of ligand binding and the relative speed of transcription. For the FMN riboswitch, it was shown that the speed of transcription relative to the kinetics of ligand binding prevents this RNA from reaching thermody-

namic equilibrium before a genetic decision must be made (*14*).

Are the Two Mg-Dependent Riboswitches the Tip of an Iceberg? Both Mg^{2+} and K^{+} are important for RNA folding *in vivo*, and Mg^{2+} has been implicated in either the stabilization or the function of a variety of ribozymes (*15*). Recently, allosteric ribozymes that respond to Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, and Cd^{2+} were generated by *in vitro* selection. They were, however, unable to discriminate among these different ligands (*16*). Interestingly, Dann, *et al.* (*8*) observed that the M-box riboswitch is a general sensor for divalent cations. However, the low intracellular concentration of the other divalent ions, here Ca^{2+} and Mn^{2+}, compared with that of Mg, prevents them from affecting this RNA element *in vivo*. Because binding pockets with different affinity for Mg^{2+} exist, it would not be surprising if RNA would form high-affinity binding pockets with a larger or smaller radius for other ions. Therefore, the idea of naturally occurring riboswitches that specifically bind other metal ions in their intracellular concentration range does not seem far-fetched.

Metal Homeostasis and the Multitude of Its Regulators. For a long time, only proteins were known to directly respond to metal ion concentrations and, therefore, to regulate metal homeostasis. These metal-responsive transcriptional regulators were classified into five families that bind a wide range of metal ions directly, thereby affecting operator binding affinity or promoter structures (reviewed in refs 6 and 17).

Therefore, the discovery of the first Mg^{2+}-responsive riboswitch in the Gram-negative bacterium *Salmonella typhimurium* in 2006 in the pioneering work of the Groisman laboratory came as a real surprise (reviewed in refs 5 and 6). This riboswitch in the 5′ untranslated region (UTR) of the *mgtA* gene, which seems to be evolutionarily conserved among some Gram-negative bacteria, acts in concert with a Mg^{2+}-sensing two-component system to measure both intracellular and periplasmic Mg^{2+} concentrations. The *mgtA* riboswitch has been characterized by extensive transcription attenuation assays and structure-probing experiments. Two regions crucial for Mg^{2+} sensing were identified (*5*). However, its mechanism of action is still elusive, because it lacks a conventional transcription terminator structure. So far, no 3D structure is available.

Small antisense RNAs are also implicated in controlling metal transport and utilization. About five years ago, the 90-nt-long RNA RyhB was shown to regulate iron homeostasis in *E. coli* (*18*). It represses post-transcriptionally the translation of iron-using proteins and ultimately affects the abundance of at least 18 transcripts encoding 56 proteins (reviewed in ref 19). RyhB transcription itself is repressed by the iron-bound Fur protein when iron is high. Under conditions of iron deprivation, Fur inactivation enables expression of iron-acquisition genes and of RyhB, which represses the expression of iron-using proteins. So, the concerted action of a metal-sensing protein and a metal-dependent small RNA (sRNA) balances minor intracellular iron fluctuations and iron usage during starvation. In *Pseudomonas aeruginosa*, two Fur-regulated sRNAs, PrrF1 and PrrF2, appear to have the same function (reviewed in ref 19). Therefore, repression of iron-using genes by sRNAs might be a common mechanism in bacteria.

In summary, bacterial metal homeostasis can be regulated at three levels: (i) at the transcriptional level by metal-binding repressor proteins, (ii) at the post-transcriptional level by antisense RNAs expressed at low metal ion concentrations, and (iii) at a cotranscriptional level by riboswitches that directly bind metal ions.

Concluding Remarks. Although first considered an oddity, Mg^{2+}-sensing riboswitches appear to be yet another widespread class of such elements in both Gram-positive and Gram-negative bacteria. Currently, the *B. subtilis mgtE* RNA aptamer may constitute a model for additional metal-sensing riboswitches that still await discovery. Although our knowledge of the regulatory repertoire of RNA is still far from complete, the elucidation of the crystal structure of the M-box and its mechanism of action is one further step toward our understanding of riboswitch function.

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